type of cellulosic chiral stationary phase (Chiralcel-OD) with a mobile phase of *n*-hexane-2-propanol (9 : 1) is used for separation of sulconazole enantiomers.

Conclusion

Since there is an enormous volume of information on the separation of antibiotics in the literature, readers should be able to find HPLC conditions for almost any antibiotic of interest. Readers are also encouraged to consult the official compendia for analysis of bulk or formulated drugs. For analysis of biological samples, the samples may be directly injected with a column switching technique instead of employing liquid–liquid or solid-phase extraction. For sensitive detection, drugs may be subjected to pre- or post-column derivatization, especially with a fluorescent chromophore. Diastereomeric derivatization is useful for analysis of chiral drugs. Mass spectrometric (MS) detection is another way to increase sensitivity. Indeed, cephem and macrolide antibiotics are analysed with HPLC-MS to detect minute amount of drugs. For cephem antibiotics, capillary HPLC has been coupled with mass spectrometric detection.

See also: **II / Chromatography: Liquid:** Derivatization; Detectors: Fluorescence Detection; Instrumentation.

Further Reading

Foster RT, Carr RA, Pasutto FM and Longstreth JA (1995) Stereospecific high-performance liquid chromatographic assay of lomefloxacin in human plasma. *Journal of Pharmaceutical and Biomedical Analysis* 13: 1243–1248.

Griggs DJ and Wise R (1989) A simple isocratic high-pressure liquid chromatographic assay of quinolones in serum. *Journal of Antimicrobial Chemotherapy* 24: 437–445.

Itoh T and Yamada H (1995) Diastereomeric β-lactam antibiotics: analytical methods, isomerization and stereoselective pharmacokinetics. *Journal of Chromatography A* 694: 195–208.

Kirschbaum JL and Aszalos A (1986) High-performance liquid chromatography. In: Aszalos A (ed.) *Modern Analysis of Antibiotics*, pp. 239–322. New York: Marcel Dekker.

Lehr KR and Damm P (1988) Quantification of the enantiomers of ofloxacin in biological fluids by high-performance liquid chromatography. *Journal of Chromatography* 425: 153–161.

Margosis M (1989) HPLC of penicillin antibiotics. In: Giddings JC, Grushka E and Brown PR (eds) *Advances in Chromatography*, pp. 333–362. New York: Marcel Dekker.

Matsuoka M, Banno K and Sato T (1996) Analytical chiral separation of a new quinolone compound in biological fluids by high-performance liquid chromatography. *Journal of Chromatography B* 676: 117–124.

Stead DA and Richards RME (1996) Sensitive fluorimetric determination of gentamicin sulfate in biological matrices using solid-phase extraction, pre-column derivatization with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *Journal of Chromatography B* 675: 295–302.

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Supercritical Fluid Chromatography

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Introduction

The analysis of antibiotics is of primary importance for drug monitoring in pharmacokinetic and health studies, as well as for the quality control of drug production and of numerous food products. As a consequence, the demand for new methods of determination of antibiotics of very different types is continuously increasing. The main methods employed for these analyses include immunoassays and chromatography, as well as various chemical techniques. Among the chromatographic methods, high performance liquid chromatography (HPLC) is the most commonly used, followed by thin-layer chromatography and gas chromatography (GC), while supercritical fluid chromatography (SFC) is still being introduced to this area of application.

In SFC the mobile phase is a fluid subjected to pressures and temperatures near or above the critical point of that fluid, to enhance and control the mobile-phase solvating power. This fact determines that the mobile-phase properties (e.g. diffusivity, density, viscosity) are intermediate between those of gases and liquids and can be varied and controlled by small changes in the pressure or temperature of the systems. The most common fluid used in SFC is carbon dioxide, which has a critical temperature of 31°C, allowing the separation of thermally labile compounds under mild conditions. In general, antibiotics are compounds with intermediate to high polarity, while

supercritical carbon dioxide only has an adequate solvating power for nonpolar compounds. For that reason, the elution of more polar solutes requires the addition of a more polar organic solvent (for example, 5–30% methanol), the so-called modifier, that increases the polarity of the mobile phase and has a solvating effect on silica-based packed columns. With this technique it is possible to separate antibiotics in complex samples at lower temperatures than GC and in shorter times than liquid chromatography. The use of low concentrations of additives in the modifier (for example, 0.1% trifluoroacetic acid and/or 0.1% triethylamine) is also used to control the separation conditions to an even greater extent, especially retention, peak shape and enantioselectivity.

SFC can be divided into two categories based on column type – open tubular and packed – with differences in selectivity, detection and need for modifier addition to the carbon dioxide characterizing the two types. Both types have been employed in the separation of drugs in very different samples. Separations of antibiotics and related compounds are best performed using packed columns, although there are some applications that do well on open tubular columns. Packed columns can be used with UV detection and a wide range of packing materials, from pure silica, to phenyl, diol, amino, octadecyl-modified silica or chiral materials such as cyclodextrins, derivatized cellulose or amylose. For these silica-based columns, the peak symmetry is improved and the retention times of the antibiotics are shortened when a modifier is added to the carbon dioxide, due to the solvating effect on the free silanol groups of the silica (cf. end-capping in HPLC). In general, the separation of antibiotics in SFC is affected by the number, location, nature and conformation of the individual functional groups, which can define the need or not, as the case may be, of a modifier and additive. Nevertheless, the determination of antibiotics by SFC is not so straightforward as other pharmaceutical separations and until now it has not been thoroughly developed.

The area that has been developed the most is probably the chiral separation of antibiotics and related compounds, where the combination of a temperature that is milder and more selective than GC and an efficiency better than HPLC results in enhanced resolution, which is especially valuable.

Another area where supercritical fluids have a niche is the monitoring of food products for antibiotic residues. This area is of increasing importance due to the concern for the effect on human health that abuse of these drugs can have. In this case, the main advantage of supercritical fluids is in the sample preparation from these complex matrices, when supercritical fluid extraction coupled to SFC can be used.

SFC versus HPLC for the Determination of Antibiotics and Related Drugs

The main problem posed in the separation of antibiotics is their broad range of structures that covers almost the whole range of organic chemistry. This includes carbohydrate, macrocyclic lactones, quinones, peptides and heterocyclic compounds, although antibiotics in general are relatively polar, nonvolatile and thermally labile drugs. For that reason, liquid chromatography has increasingly been chosen as the method of analysis, and SFC is gaining greater acceptance with the extended use of packed columns combined with organic modifiers and additives that allow the separation of the more polar solutes.

GC commonly provides the highest resolution in the shortest analysis time, but it also requires high temperatures and often derivatization of the drugs. HPLC methods have lower resolution and longer analysis times. The SFC technique can provide the same resolution as GC and short run times, but with the added benefit that it does not need high temperatures (**Figure 1**). Typical temperatures are as low as 50–80°C in packed-column SFC.

The packed columns used for SFC of polar drugs are similar to the columns used in HPLC, although back-pressure is not a problem in SFC, allowing columns to be coupled in series to achieve high resolution systems, even for polar analytes. When it comes to detection, the commonest systems for antibiotics determination are UV (**Figure 2**). or mass spectrometry (MS). In comparing packed-column SFC and LC, the ultraviolet detector can be operated at lower wavelengths in SFC, because of the lack of background absorbance from the solvent and the mass spectrometric ionization techniques work best with volatile mobile phases also favouring SFC (**Table 1**).

Characteristics of the Separation of Antibiotics using Supercritical Fluids

The main properties of SFC which significantly affect antibiotic separation are related to the high solvating power of supercritical fluids and their low viscosity, which yields high resolution power and throughput. This fact has two main consequences on this type of separation. Firstly, as already pointed out above, compounds like antibiotics can be analysed at lower temperatures than in gas chromatography, and in shorter times than in liquid chromatography, as a result of good solvating capacity. Secondly, SFC is able to resolve complex mixtures of not very volatile compounds, allowing the direct injection of samples

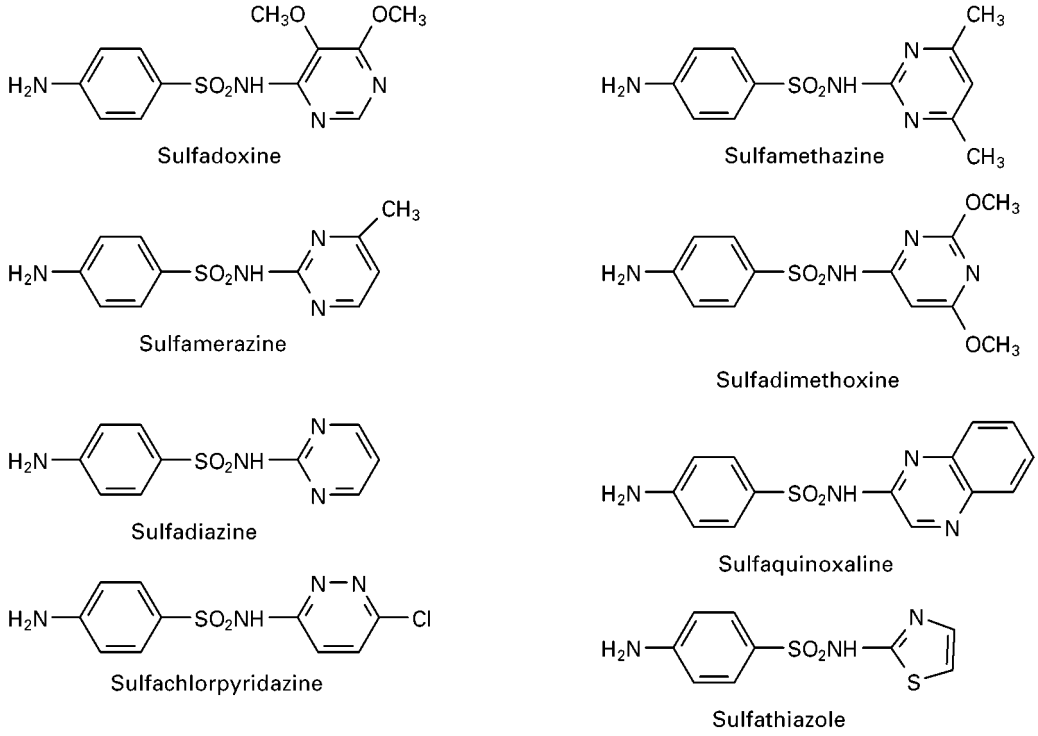

Figure 1 Structures of eight sulfonamides determined by SFC (see chromatogram shown in Figure 2).

that contain antibiotics with little or no sample pretreatment.

Some antibiotics may be degraded or lost during exposure to light, heat or extreme values of pH. In SFC, all these factors are avoided, providing separation under mild conditions that preserves the integrity of the sample.

Antibiotic determination presents some difficulties due to the complexity of the sample matrix and the relatively low concentration of the antibiotics in these samples. The whole procedure, including extraction and fractionation, is not only time-consuming and prone to error, but may degrade labile antibiotics and create artefacts. Consequently, new approaches have been developed in the last few years that avoid several or all of these questionable sample preparation steps by using multidimensional systems or even direct injection in SFC.

The analysis of antibiotics in human serum has been performed with both open tubular and packed-column SFC. With open tubular columns, it is possible to determine the antibiotic content with a simple liquid–liquid extraction. The use of a flame ionization detector has, however, not demonstrated satisfactory detection limits to date. Better results have been obtained with SFC and mass spectrometric detection, which thus provides a very useful method for the determination of low levels of impurities in macrolide antibiotics, and presents an alternative approach to several LC-MS methods.

Online SFE-SFC for the Analysis of Antibiotics

Online supercritical fluid extraction (SFE)-SFC can be used for the analysis of antibiotics in complex samples, resulting in time savings and less exposure to organic solvents. Multidimensional systems take advantage of two orthogonal separation techniques with complementary characteristics, for example one extraction and one chromatographic step, where the first step is aimed at producing a clean and undiluted sample containing the compounds of interest, and the second step provides a high resolution separation of the target analytes.

The main advantages of these online systems is that a fast and automatic sample preparation reduces or avoids the errors of the manual steps. Also, solvent consumption is less, which reduces toxic hazards and disposal costs. As is often the case in chromatography, the largest source of error in the quantitative analysis of antibiotics and the most time-consuming steps occur in the sample preparation and extraction stages.

Supercritical fluid techniques have a number of advantages for use in multidimensional chromatographic

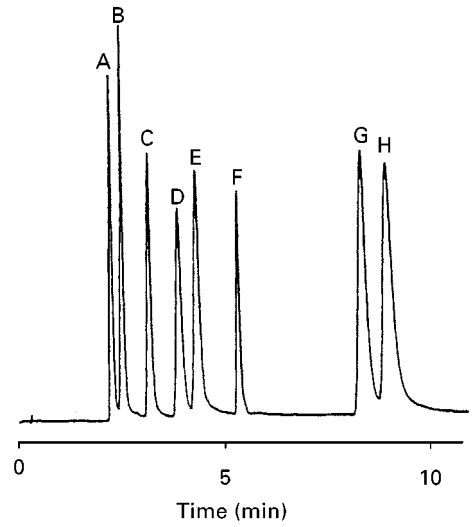

Figure 2 Chromatogram obtained from SFC of a mixture of sulfonamides with UV (wavelength 270 nm). Peak identification: A, sulfadoxine; B, sulfamethazine; C, sulfamerazine; D, sulfadimethoxine; E, sulfadiazine; F, sulfaquinoxaline; G, sulfachlorpyridazine; H, sulfathiazole. Chromatographic conditions: column packed with 5 μm particle amino-bonded Spherisorb (100 × 4.6 mm i.d.), column temperature 90°C, CO_2 flow rate of 4 mL min^{-1}, pressure 361 bar. Mobile phase was CO_2 modified initially with 15% methanol and after 4 min with 25% methanol. Reproduced with permission from Perkins JR, Games DE, Startin JR and Gilbert JJ (1991) Analysis of sulfonamides using supercritical fluid chromatography and supercritical fluid chromatography–mass spectrometry. *Journal of Chromatography* 540: 239.

systems. The commonest multidimensional system, LC-GC, is limited to the determination of thermally stable and volatile solutes, while SFC can substitute the first fractionation step, as well as the second step of high resolution chromatography. In the case of SFE-SFC, the transfer is performed without changes in the mobile phase, which minimizes losses of analytes and reduces technical complexity

Analysis of Aqueous Matrices

Recently, new methods for the direct injection of water samples on to an adsorbent with solvent venting and online SFE-SFC of the target solutes have been developed.

In this procedure, the liquid sample is introduced in the SFE cell filled with a suitable adsorbent, which retains the solutes while the aqueous solvent is vented with an inert gas. The venting of the water improves the performances and flexibility of both the separation and the detection steps. After elimination of solvent, the analytes are extracted with supercritical carbon dioxide, and focused in a cryogenic trap, providing a solute enrichment before automatic online injection into the SFC column. In addition to its speed, this method provides a preconcentration step for the analysis of trace levels of residues in biofluid samples and also allows class-selective extractions based on the tuneable polarity of the extracting agent, which thus represents an additional clean up stage and further reduces interface from the matrix.

This coupling has to date been applied to separations in open tubular columns of compounds in water samples, and may provide a breakthrough development for the future, with the use of packed capillary column SFC-MS for polar analytes. Although this method has not yet been applied to the determination of antibiotics, it could provide an automatic way of analysing drugs in biological fluids directly, i.e. with no separate extraction step.

Analysis of Antibiotic Residues in Food

Antibiotics have been used in animal feed for several decades to control infections and promote growth (**Figure 3**). Recently, increasing concern about antibiotic resistance has led to the prohibition of the use of some antibiotics for this purpose in several countries. Consequently, there is a growing interest in new and improved methods of analysis for antibiotics and their residues in food, and LC-MS is a common technique used to achieve this goal.

In solid and heterogeneous matrices such as food, sample preparation is the most time-consuming step in the routine determination of analytes in trace amounts, for instance, antibiotic residue levels. It remains the largest source of error in quantitative analytical methods. For this reason, the development of methods with less sample pretreatment requirements and with the possibility of automation is desirable.

For this application, SFC in combination with the universal flame ionization detector, or with highly sensitive mass spectrometric detection, shows a good balance between high resolution and good sample throughput, that can minimize the need for sample clean-up and be an optimal procedure in specific cases. An example of separation of veterinary antibiotics by SFC with UV detection prior to online SFC-MS can be seen in **Figure 4**.

An alternative approach is the online coupling of SFE and SFC for solid or semi-solid samples, allowing the extraction of the fraction of interest and the online transfer of the solutes from the liquid or solid matrix directly to the chromatograph, reducing solvent usage and the need for clean-up.

Chiral Separation of Antibiotics

The stereochemistry of an antibiotic is a prominent issue in the development, approval and clinical use of

Table 1 Determination of antibiotics by supercritical fluid chromatography

Column type	*Sample*	*Comments*	*Detector*	*Reference*
Packed (1 mm i.d., 5 μm particles) and open tubular (50 μm i.d.) columns	Sulfonamides and tetracyclines	100°C 8% Isopropanol as modifier	UV	Schmidt S, Blomberg, LG and Campbell ER (1988) *Chromatographia* 25: 775
Packed column 150 × 4.6 mm 5 μm particle amino silica	Erythromycin A Cephalosporins	65°C 2–8% Methanol as modifier	MS UV	Lane SJ (see Markides and Lee, 1988)
Packed column 100 × 4.6 mm 5 μm particle amino-bonded Spherisorb	Sulfonamides, veterinary drugs	75–90°C 15–25% Methanol as modifier	MS UV	Perkins JR, Games DE, Startin JR and Gilbert JJ (1991) *Journal of Chromatography* 540: 239
Packed column 250 × 4.6 mm 10 μm particle Chiralcel OB	Lactams	25°C Chiral separation	Diode array	Caude and Macaudiere (see Markides and Lee, 1988)
Open tubular 10 m × 50 μm i.d. SB-biphenyl-100	Cyclosporine A FK 506 (Tacrolimus) Rapamycin	70°C Whole blood extracts	FID	Wong *et al.* (1994) *Journal of Liquid Chromatography* 17: 2093
Open tubular 5 m × 50 μm i.d. DB-5	Macrolide antibiotic Midecamycin A_1	100°C Standard solutions	MS	Ramsey *et al.* (1995) *Analytical Proceedings* 32: 455
Different packed columns 250 × 4.6 mm 5 μm particles	Sulfonamides	50–100°C Great influence of temperature on resolution	UV	Combs MT, Ashraf-Khorassani M and Taylor LT (1997) *Journal of Chromatographic Science* 35: 176

This summary is not intended to be a comprehensive review of all antibiotic separations by SFC; it aims to provide general information on the main applications in this field.

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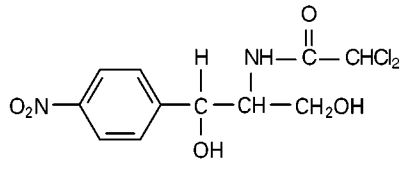
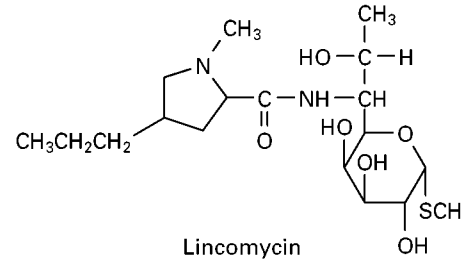
these drugs. For the separation of enantiomers, SFC with chiral stationary phases is very convenient due to its high resolution and relatively low analysis temperature.

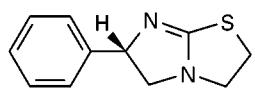
Chiral separations in SFC can be carried out using open tubular columns with immobilized cyclodextrins or, more recently, by packed columns with most of the same phases commonly used in LC, since the



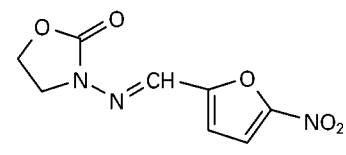
Chloramphenicol



Lincomycin



Levamisole



Furazolidone

Figure 3 Structures of some veterinary antibiotics analysed by SFC.

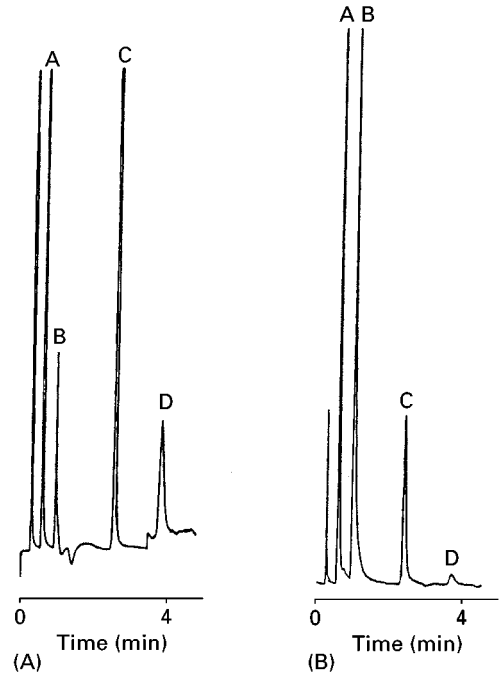


Figure 4 Chromatograms obtained from SFC of a mixture of veterinary antibiotics with UV (A, wavelength 215 nm; B, wavelength 230 nm). Peak identification: A, levamisole; B, furazolidone; C, chloramphenicol; D, lincomycin. Chromatographic conditions: column packed with 5 μm particle amino-bonded Spherisorb (100 × 4.6 mm i.d.), column temperature 75°C, CO_2 flow rate of 4 mL min^{-1}, pressure 351 bar. Mobile phase was CO_2 modified with 15% methanol. Reproduced with permission from Perkins *et al.* (1991).

chiral selector is covalently bound to the packing material. The latter method frequently requires the addition of a modifier and is more common in the separation of drugs.

Several examples of the SFC of antibiotic enantiomers can be found in the literature, although the technique is not as commonly used as LC. Packed columns with chiral stationary phases are normally employed with carbon dioxide modified with methanol or ethanol as mobile phase, under supercritical or subcritical conditions (i.e. at room temperature). Further applications can be expected from the use of packed capillary columns for chiral separations, which may provide better resolution and shorter analysis times than the equivalent LC separation.

Future Developments

Current developments in new types of columns, equipment and detectors for SFC show that this technique has great potential for expansion and will achieve a broader use in the future with the advent of new instruments for packed capillary columns and adaptation of the routine use of MS-detectors, which will be very valuable in the accurate determination of high and low concentration of antibiotics in samples where high resolution and mild conditions are imperative.

It is becoming more frequent to use solvents under subcritical conditions, which is blurring the boundary between SFC and LC – a fact that is already being used in chiral separations and will probably become more frequent in separations of antibiotics and related drugs.

Another probable source of improvement is the use of new detectors with higher sensitivity than UV and flame ionization detection and at the same time compatible with the use of modifiers in packed capillary column SFC. An example is the new amperometric detectors which avoid the problems observed in the quantitation of some drugs by SFC.

The development of new generations of commercial equipment for SFE-SFC and SFC-MS that are more user-friendly and robust than the present ones would also contribute to a wider use of this technology in quality control and research of pharmaceutical compounds.

Further Reading

Agarwal VK (ed.) (1992) *Analysis of Antibiotic/Drug Residues in Food Products of Animal Origin*. New York: Plenum Press.

Ahuja S (ed.) (1992) *Chromatography of Pharmaceuticals*. Washington, DC: American Chemical Society.

Jinno K (ed.) (1992) *Hyphenated Techniques in Supercritical Fluid Chromatography and Extraction*. Amsterdam: Elsevier.

Lee ML and Markides KE (eds) (1990) *Analytical Supercritical Fluid Chromatography and Extraction*. Provo, UT: Chromatography Conferences.

Markides KE and Lee ML (1988) *SFC Applications*. *Provo*, UT: Brigham Young University Press.

Medvedovici A, Sandra P, Toribio L and David F (1997) Chiral packed column subcritical fluid chromatography on polysaccharide and macrocyclic antibiotic chiral stationary phases. *Journal of Chromatography A* 785: 159.

Perkins JR, Games DE, Startin JR and Gilbert J (1991) Analysis of veterinary drugs using supercritical fluid chromatography and supercritical fluid chromatography–mass spectrometry. *Journal of Chromatography* 540: 257.

Señoráns FJ and Markides KE (2000) On line SFE-SFC for the analysis of fat soluble vitamins and other lipids from water matrices. In: Williams JR (ed.) *Methods in Molecular Biology*: *Supercritical Fluid Methods and Protocols*. Totowa, NJ: Humana Press.

Xie LQ, Markides KE and Lee ML *et al.* (1993) Bioanalytical application of multidimensional open tubular column supercritical fluid chromatography. *Chromatographia* 35: 363.